AbiV, a Novel Antiphage Abortive Infection Mechanism on the Chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363[∇]

Jakob Haaber,¹ Sylvain Moineau,² Louis-Charles Fortier,^{2†} and Karin Hammer^{1*}

Center for Systems Microbiology, DTU Biosys, Technical University of Denmark, DK-2800 Lyngby, Denmark,¹ and
Département de biochimie et de microbiologie, Faculté des sciences et de génie, Groupe de recherche en
écologie buccale, Faculté de médecine dentaire, Félix d'Hérelle Reference Center for
Bacterial Viruses, Université Laval, Québec, Canada G1V 0A6²

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Insertional mutagenesis with pGhost9::ISS1 resulted in independent insertions in a 350-bp region of the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363 that conferred phage resistance to the integrants. The orientation and location of the insertions suggested that the phage resistance phenotype was caused by a chromosomal gene turned on by a promoter from the inserted construct. Reverse transcription-PCR analysis confirmed that there were higher levels of transcription of a downstream open reading frame (ORF) in the phage-resistant integrants than in the phage-sensitive strain *L. lactis* MG1363. This gene was also found to confer phage resistance to *L. lactis* MG1363 when it was cloned into an expression vector. A subsequent frameshift mutation in the ORF completely eliminated the phage resistance phenotype, confirming that the ORF was necessary for phage resistance. This ORF provided resistance against virulent lactococcal phages belonging to the 936 and c2 species with an efficiency of plaquing of 10^{-4} , but it did not protect against members of the P335 species. A high level of expression of the ORF did not affect the cellular growth rate. Assays for phage adsorption, DNA ejection, restriction/modification activity, plaque size, phage DNA replication, and cell survival showed that the ORF encoded an abortive infection (Abi) mechanism. Sequence analysis revealed a deduced protein consisting of 201 amino acids which, in its native state, probably forms a dimer in the cytosol. Similarity searches revealed no homology to other phage resistance mechanisms, and thus, this novel Abi mechanism was designated AbiV. The mode of action of AbiV is unknown, but the activity of AbiV prevented cleavage of the replicated phage DNA of 936-like phages.

Lactococcus lactis starter cultures are used for the production of fermented dairy products worldwide. However, bacteriophage infection of the added starter cultures may lead to fermentation delays or even halt the process. Despite considerable progress over the past few decades that has led to improved phage control measures, this natural phenomenon still remains a significant risk to the dairy industry (2, 14, 55). Three groups of lactococcal phages cause the vast majority of milk fermentation failures, namely, members of the 936 and c2 species, which display great intraspecies homogeneity but are genetically distinct from each other (12, 30, 50), and phages belonging to the P335 species, which exhibit much greater genomic mosaicism (12, 44). To survive infection by these diverse phages, *L. lactis* strains possess a wide variety of resistance mechanisms. Lactococcal phage defense systems are classified into four general groups depending on the step of the phage lytic cycle that they inhibit (39). The members of two groups either prevent phage adsorption or block DNA ejection (55), and the members of the other two groups are intracellular antiphage hurdles, namely, restriction/modification systems and abortive infection (Abi) mechanisms. The Abi mechanisms

have been described by different workers (for reviews, see references 14 and 73) and are arguably the most efficient mechanisms.

To date, 22 lactococcal Abi mechanisms have been isolated, characterized, and designated AbiA through AbiZ (14, 25). Remarkably, most of these mechanisms appear to have a distinct mode of action, although they do share some common features. These features include reduction of the burst size, reduction of the efficiency of plaquing (EOP) (18, 26), and reduction of the efficiency of formation of centers of infection, as well as death of the infected cells (73). The Abi phenotype is usually mediated by a single gene, although in a few cases (AbiE, AbiG, AbiL, AbiT, and AbiU) the system consists of two genes (9, 18, 19, 31, 62). There is some level of sequence similarity between different Abi proteins (14), but in general, these proteins and their genes show little similarity to other proteins and genes in databases. This finding is in agreement with the activity of the proteins against specific phages or phage groups, as well as the particular mechanistic models. Indeed, the Abi mechanisms of wild-type *L. lactis* strains that have been characterized have been shown to disable members of one, two, or more lactococcal phage groups, although there is a tendency toward broader efficacy against 936 phages (14), which represent the group that causes most dairy fermentation failures. The general effects on the phage lytic cycle have been revealed for most Abi mechanisms, although in most cases much more characterization is required. For example, AbiA, AbiF, AbiK, AbiP, and AbiT were shown to interfere with DNA replication (9, 23, 27, 31, 36), while AbiB, AbiG, and

* Corresponding author. Mailing address: Center for Systems Microbiology, DTU Biosys, Matematiktorvet, Bldg. 301, Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 45 45 25 24 96. Fax: 45 45 93 28 09. E-mail: kha@bio.dtu.dk.

† Present address: Département de microbiologie et d'infectiologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, 3001 12e Ave Nord, Sherbrooke, Québec, Canada J1H 5N4.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics ^a	Reference or source
<i>L. lactis</i> strains		
IL1403	<i>L. lactis</i> subsp. <i>lactis</i> , host for 936-like phages	8
MB112	<i>L. lactis</i> subsp. <i>cremoris</i> MG1363, <i>Δupp</i> , host for 936- and c2-like phages	51
MG1363	<i>L. lactis</i> subsp. <i>cremoris</i> , host for 936-like and c2-like phages	33
SMQ-86	<i>L. lactis</i> subsp. <i>cremoris</i> , host for P335-like phages, <i>Erm</i> ^r	27
JH-20	MB112(pJH2), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-22	IL1403(pJH2), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-23	SMQ-86(pJH2), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-24	MB112(pJH3), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-32	MB112 with pGhost9::ISS1 inserted into Lin at position 1962; <i>Erm</i> ^r <i>Abi</i> ⁺	This study
JH-46	MB112 with pGhost9::ISS1 inserted into Lin at position 1962; <i>Erm</i> ^r <i>Abi</i> ⁺	This study
JH-47	MB112 with pGhost9::ISS1 inserted into Lin at position 2296; <i>Erm</i> ^r <i>Abi</i> ⁺	This study
JH-48	MB112 with pGhost9::ISS1 inserted into Lin at position 2240; <i>Erm</i> ^r <i>Abi</i> ⁺	This study
JH-49	JH-32 cured of pGhost9, <i>ISS1</i> ⁺ <i>Abi</i> ⁺	This study
JH-50	JH-46 cured of pGhost9, <i>ISS1</i> ⁺ <i>Abi</i> ⁺	This study
JH-51	JH-47 cured of pGhost9, <i>ISS1</i> ⁺ <i>Abi</i> ⁺	This study
JH-52	JH-48 cured of pGhost9, <i>ISS1</i> ⁺ <i>Abi</i> ⁺	This study
JH-53	MB112(pJH6), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-54	MB112(pLC5), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
JH-79	MG1363(pJH2), <i>Cam</i> ^r <i>Abi</i> ⁺	This study
<i>E. coli</i> strains		
EC1000	RepA ⁺ MC1000, <i>Km</i> ^r	45
JH-56	EC1000(pJH7), <i>Erm</i> ^r	This study
JH-57	EC1000(pJH8), <i>Erm</i> ^r	This study
JH-58	EC1000(pJH9), <i>Erm</i> ^r	This study
JH-59	EC1000(pJH10), <i>Erm</i> ^r	This study
JH-62	M15(pJH11), <i>Km</i> ^r <i>Amp</i> ^r	This study
M15	pREP4, <i>Km</i> ^r , used for cloning of pQE-70 His-tagged vector	Qiagen
MC1061	Laboratory strain, cloning host	54
TOP10F'	Chemically competent cells from a TOPO TA cloning kit	Invitrogen

^a Lin, GenBank accession number AF324839 sequence; *Abi*⁺, phage-resistant phenotype; *Abi*[−], phage-sensitive phenotype; *Amp*^r, ampicillin resistance; *Cam*^r, chloramphenicol resistance; *Erm*^r, erythromycin resistance; *Km*^r, kanamycin resistance.

AbiU affected RNA transcription (15, 18, 63). *AbiC* was shown to cause limited major capsid protein production (59), whereas *AbiE*, *AbiI*, and *AbiQ* affected phage packaging (26). *AbiD1* was found to interfere with a phage RuvC-like endonuclease (6, 7), and the presence of *AbiZ* caused premature lysis of the infected cells (25).

Most *Abi* systems are encoded on plasmids, some of which are conjugative, thereby enabling lateral transfer of the resistance mechanisms to phage-sensitive *L. lactis* strains and subsequent industrial use of the phage-resistant derivatives. Usually, *Abi* mechanisms provide a much stronger phage resistance phenotype when they are plasmid encoded (due to a higher gene copy number) than when they are chromosomally encoded, which may explain why so few lactococcal *Abi* genes are in bacterial genomes (14, 15, 20, 64, 66).

Natural *Abi* mechanisms have been used extensively for protection of industrial starter cultures (16, 28). Not surprisingly, the added selective pressure has led to the emergence of phage mutants capable of overcoming the resistance barriers (16, 28). Therefore, isolation of novel phage resistance mechanisms that can be used alone or in combination with other mechanisms to provide increased phage protection to starter cultures is highly desirable (55).

We report here a novel lactococcal *Abi* system, designated *AbiV*, which is chromosomally encoded and effective against virulent phages belonging to the 936 and c2 species. This novel system was discovered using insertional mutagenesis of the

laboratory workhorse strain *L. lactis* MG1363. Although insertional mutagenesis is usually used in loss-of-function studies to characterize the effect of a genetic knockout (24, 49, 52), our data indicated that the expression of *AbiV* was turned on by insertion of pGhost9::ISS1. Microbiological and molecular characterization of *AbiV* is also described below.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. Bacterial strains used in this study are listed in Table 1, and bacteriophages and plasmids are listed in Table 2. Bacteriophages sk1 and jj50 were kindly provided by F. K. Vogensen (University of Copenhagen). *L. lactis* strain MB112 was used as the host for selection of phage-resistant integrants. This strain is a derivative of *L. lactis* subsp. *cremoris* MG1363 and has a mutation in the *upp* gene. This mutation makes *L. lactis* MB112 resistant to fluorouracil, which provided an additional marker to confirm that the phage-resistant integrants were derived from the parental strain (51). *Escherichia coli* was grown at 37°C in LB medium (70). *L. lactis* was grown in M17 medium (74) supplemented with 0.5% glucose (GM17). When fluorouracil resistance was tested, *L. lactis* strains were grown in SA medium supplemented with 0.5% glucose (38). Most lactococci were grown at 30°C; the exceptions were strains containing the thermosensitive vector pGhost9::ISS1, which were grown at 28 and 37°C to allow replication and integration, respectively, of the vector. When *L. lactis* was grown at temperatures above 30°C, NaCl (1%) was added to M17 medium (41). In phage infection experiments, 10 mM CaCl₂ was added to plates or to the medium. When appropriate, antibiotics were added at the following concentrations: for *E. coli*, 100 μg ml^{−1} of ampicillin, 10 μg ml^{−1} of chloramphenicol, and 150 μg ml^{−1} of erythromycin; and for *L. lactis*, 3 μg ml^{−1} of chloramphenicol, 3 μg ml^{−1} of erythromycin, and 0.3 μg ml^{−1} fluorouracil.

TABLE 2. Phages and plasmids used in this study

Phage(s) or plasmid	Relevant characteristics ^a	Reference(s) or source
Phages		
bIL170	Small isometric headed, 936 species	17
jj50	Small isometric headed, 936 species	40, 50
p2	Small isometric headed, 936 species	60
P008	Small isometric headed, 936 species	46, 50
sk1	Small isometric headed, 936 species	13
712	Small isometric headed, 936 species	50
c2	Prolate headed, c2 species	71
bIL67	Prolate headed, c2 species	72
eb1, ml3	Prolate headed, c2 species	60
ul36	Small isometric headed, P335 species	43, 57
ul36.k1t1	Small isometric headed, P335 species	44
φ31, φ50	Small isometric headed, P335 species	1
P335	Small isometric headed, P335 species	11
Q30, Q33	Small isometric headed, P335 species	56
Plasmids		
pCI372	Cloning vector for <i>E. coli</i> and <i>L. lactis</i> , Cam ^r	34
pCR II-TOPO	Cloning vector, Amp ^r	Invitrogen
pGhost9::ISS1	Temperature-sensitive vector used for mutagenesis, Erm ^r	49
pJH2	Position 1021 to 2320 fragment cloned in PstI site of pLC5, Cam ^r	This study
pJH3	pJH2 cut in ClaI site, filled with the Klenow fragment, <i>abiV</i> frameshift mutation; Cam ^r	This study
pJH6	Position 1021 to 2320 fragment cloned in PstI and XbaI sites of pCI372	This study
pJH7	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-32	This study
pJH8	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-46	This study
pJH9	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-47	This study
pJH10	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-48	This study
pJH11	Position 1273 to 1875 fragment cloned in pQE-70, C-terminal His tag, Amp ^r	This study
pLC5	<i>E. coli</i> - <i>L. lactis</i> shuttle expression vector, promoter in front of PstI, Cam ^r	This study
pQE-70	Cloning vector for C-terminal His tagging of proteins, Amp ^r	Qiagen

^a The fragment mentioned is a fragment of the GenBank accession number AF324839 sequence. Amp^r, ampicillin resistance; Cam^r, chloramphenicol resistance; Erm^r, erythromycin resistance; Km^r, kanamycin resistance.

Insertional mutagenesis with pGhost9::ISS1 and isolation of phage-resistant mutants. Phage-resistant derivatives of *L. lactis* MB112 were obtained by insertional mutagenesis using the vector pGhost9::ISS1, essentially as described by Maguin et al. (49). Subsequent cloning of the flanking chromosomal DNA was also performed as described by Maguin et al. (49). To ensure isolation of phage-resistant integrants from distinct events, mutagenesis was performed with three independent cultures of *L. lactis* MB112 containing pGhost9::ISS1. These cultures were grown overnight at 28°C in GM17 containing erythromycin. The cultures were then diluted 100-fold in GM17 and grown for 2.5 h at 28°C to obtain exponential growth. The cells were incubated at 37°C for 2.5 h to allow plasmid integration. The cultures were diluted again 10,000-fold in 50 ml of GM17 supplemented with erythromycin and incubated overnight at 37°C to allow phenotypic expression. These overnight cultures were inoculated (1%) into fresh GM17 with erythromycin and incubated at 37°C. When the exponential growth phase was reached, aliquots were mixed with the virulent lactococcal phage sk1 (multiplicity of infection [MOI], >1). Following 10 min of incubation at 37°C, the infected cells were plated on GM17 plates containing erythromycin, NaCl (1%), and CaCl₂ (10 mM) and incubated for 2 days at 37°C. The frequency of phage-resistant mutants was calculated, and 100 mutants were selected at random from the three independent cultures. The phage-resistant phenotype of these mutants was verified by cross-streaking with virulent phage sk1 at 37°C. Finally, curing the phage-resistant mutants of the pGhost9::ISS1 plasmid, leaving a single copy of ISS1 at the integration site, was performed as described by Maguin et al. (49).

RNA isolation and purification and RT-PCR analysis. Overnight cultures were diluted 100-fold and grown to an optical density at 600 nm of 0.5 at 37°C. Aliquots (2 ml) were harvested by quick centrifugation (20,000 × g, 30 s), and each pellet was resuspended in a solution of 0.5 M sucrose with 60 mg ml⁻¹ lysozyme. Following incubation (37°C, 15 min), the cells were pelleted and resuspended in 1 ml TRIzol reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instructions. Prior to reverse transcription (RT)-PCR, the RNA samples were treated with a DNase-based TURBO DNA-free kit (Applied Biosystems).

RT-PCR was carried out using a RevertAid First Strand cDNA synthesis kit

(Fermentas) as recommended by the manufacturer. As a control, the RT-PCR procedure was carried out without reverse transcriptase to ensure that there was no contaminating DNA in the RNA samples.

Phage assays. Propagation of phages was performed as described by Emond et al. (27). Determination of the titers of phage lysates (42), the EOP (71), and cross-streaking assays (69) were performed as described previously. Adsorption assays were performed as described elsewhere (71), except that 5 min of incubation was used instead of 15 min. Cell survival (5) was assayed using an MOI of 5. One-step growth curve experiments and center of infection assays (59) were performed using MOIs of 0.2 and 0.5, respectively. The efficiency of formation of centers of infection and the burst size were calculated as previously reported (59).

Phage DNA replication. Replication of phage DNA was monitored by performing a time course experiment (35). Briefly, total DNA of *L. lactis* cells was isolated at 10-min intervals from cultures infected with reference phage p2 (MOI, 2), which is closely related to phage sk1. Total DNA was digested with EcoRV and heated (65°C, 10 min) prior to gel electrophoresis, which allowed identification of resolved *cos* sites that were used to distinguish concatemeric and mature phage DNA.

Phage adsorption and DNA ejection assays using SYBR gold staining. Visualization of phage DNA by labeling with the fluorescent dye SYBR gold was performed as described by Noble and Fuhrman (61), with the following modifications. The original SYBR gold solution was diluted 1,000-fold. The phage lysate was treated with 1 µg ml⁻¹ DNase and RNase for 30 min at 37°C and then stained with the diluted SYBR gold (final concentration, 2.5% [vol/vol]). The mixture was incubated for at least 12 h at 4°C in the dark. One microliter of the labeled phage lysate was mixed with 1 µl of an exponentially growing cell culture and visualized using a Zeiss Axioplan epifluorescence microscope.

Rescue cloning and sequencing of the flanking chromosomal DNA. Chromosomal DNA was analyzed for six phage-resistant integrants. The HindIII-digested DNA was purified, diluted to favor self-ligation, and ligated as described by Maguin et al. (49). The ligation mixture was electroporated into *E. coli* EC1000 (45) and plated on LB agar with erythromycin. Plasmid DNA containing

FIG. 1. Localization of *orf1* (*abiV*) on the chromosome of *L. lactis* MG1363. The numbers indicate nucleotide positions in the GenBank accession number AF324839 sequence. (A) *L. lactis* strains with the transposon-containing vector pGhost9::ISS1 or cured of pGhost9 and thus containing a solitary ISS1 sequence inserted into the chromosome. The numbers and arrows indicate the positions and directions of the inserts. (B) *L. lactis* strains with the cloned DNA fragment (bp 1021 to 2320) including *orf1*. The arrows indicate promoter P_{59} , and the × in JH-24 indicates the position of the frameshift mutation introduced into this strain.

the rescued pGhost9::ISS1 plasmid and the flanking chromosomal DNA was isolated from *E. coli* cells (strains JH-56 to JH-59). Chromosomal DNA flanking the ISS1 element was sequenced using primer 5'-GAAGAAATGGAACGCT C-3' annealing to the ISS1 sequence. The procedure described above was successful for four of the six integrants.

DNA isolation and manipulation. Plasmid DNA was isolated from *L. lactis* using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's recommendations, except that lysozyme (15 mg ml⁻¹) was added to buffer P1 and the lysis solution with the resuspended cells was incubated at 37°C for 30 min before the rest of the protocol was performed. Restriction enzymes, T4 DNA ligase, and the Klenow fragment (Fermentas) were used according to the manufacturer's instructions. Electroporation of *L. lactis* was performed as described previously (37, 58). The DNA fragment corresponding to nucleotides 1021 to 2320 in the GenBank accession number AF324839 sequence (Fig. 1) was first cloned into the *E. coli* pCR II-TOPO vector (Invitrogen) using TOP10F' cells prior to cloning into the *E. coli*-*L. lactis* shuttle vectors pCI372 and pLC5. For protein purification, the DNA fragment corresponding to nucleotides 1273 to 1875 in the GenBank accession number AF324839 sequence was cloned into the His-tagged vector pQE-70 (Qiagen) as recommended by the manufacturer to create pJH11 in *E. coli* strain JH-62.

Protein purification and gel filtration. A C-terminally His-tagged protein was purified according to the manufacturer's instructions (Qiagen). Gel filtration was carried out using a HiPrep 16/60 Sephacryl S-300 high-resolution column from GE Healthcare, 150 µg of AbiV, and a flow rate of 0.2 ml min⁻¹. The column was connected to a Shimadzu high-performance liquid chromatography system. The solvent delivery module used was an LC-10ADvp module, and the detector used was an SPD-M10Avp detector (280 nm). The mobile phase contained 50 mM Tris-HCl and 200 mM NaCl (pH 7.6). A Bio-Rad protein standard (catalog no. 151-1901) was used to determine the molecular weight of the purified protein. Each run lasted 800 min. The molecular weight was estimated by determining the mean of four different trials.

Construction of expression vector pLC5. The low-copy-number vector pGKV259 (76) was digested with PstI (site located downstream from the P_{59} promoter), which was followed by gel purification. Two complementary oligonucleotides (5'-TGGATCAAAGGAGGTCCTGCA-3' and 5'-GGACCTCCT TTGGATCCATGCA-3') were annealed together (70) to create a double-stranded linker with PstI-compatible sticky ends. This linker also contained a

unique BamHI site and a ribosome-binding site (RBS) (5'-AGGAGG-3'). The linker was inserted into the PstI site of pGKV259, and the ligation mixture was transformed into *E. coli* MC1061. Transformants were selected on LB plates containing 20 µg ml⁻¹ of chloramphenicol. Positive clones with the linker inserted in the right direction were identified by colony PCR, and clones were confirmed by sequencing. Upon introduction of the linker into pGKV259, the PstI site on the 5' side of the linker was disrupted, whereas the site on the 3' side was conserved. Thus, a unique PstI site was created 8 bp downstream from the RBS. Cloning of a DNA fragment having an ATG start codon into the PstI site of pLC5 enabled efficient transcription from the P_{59} promoter and translation from the introduced RBS.

DNA and protein analyses. Sequence similarity searches in databases were performed using BLAST (3) at the NCBI website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Searches for helix-turn-helix motifs were performed using the website <http://npsa-pbil.ibcp.fr/>. The molecular weights and pIs of the investigated proteins were estimated using the Protein Calculator at the website <http://www.scripps.edu/~cdputnam/protcalc.html>. Searches for transmembrane domains and signal peptide motifs were performed using the predictor websites <http://www.cbs.dtu.dk/services/TMHMM-2.0/> and <http://www.cbs.dtu.dk/services/SignalP/>.

RESULTS

Isolation of phage-resistant mutants of *L. lactis* subsp. *cremoris* MG1363 using insertional mutagenesis. The laboratory strain *L. lactis* subsp. *cremoris* MB112 (MG1363Δ*upp*) (51) is sensitive to several virulent phages belonging to the 936 and c2 species. To identify genes involved in the sensitivity of *L. lactis* MG1363 to 936 phages, mutant cells resistant to phage sk1 were isolated after insertional mutagenesis of the host. Insertional mutagenesis was performed with three cultures, which resulted in isolation of three independent insertion libraries. Selection for resistance to virulent lactococcal phage sk1 was performed for all three libraries (MOI, >1). The frequency of

phage-resistant colonies was $6.2 \times 10^{-6} \pm 2.6 \times 10^{-6}$, which is 100-fold higher than the frequency of spontaneous phage-resistant mutants obtained from wild-type cultures ($5.9 \times 10^{-8} \pm 3.0 \times 10^{-8}$).

Identification of a chromosomal gene involved in phage resistance. Six confirmed phage-resistant mutants (two mutants randomly selected from each of the three independent cultures) were chosen for plasmid rescue experiments. In four cases it was possible to isolate the inserted plasmid along with a piece of flanking chromosomal DNA (pJH7 to pJH10). Sequence analysis revealed insertions in the same 350-bp region on the chromosomes of the four phage-resistant mutants. The insertions were located in the intergenic region between two genes (designated *orf1* and *trans*) or in the 3' end of the *trans* gene (Fig. 1A). The inserts were located at nucleotides 1962 (for phage-resistant mutants JH-32 and JH-46), 2240 (JH-48), and 2296 (JH-47). The nucleotide positions are based on the GenBank accession number AF324839 sequence. Strains JH-32 and JH-46 originated from the same mutation library and may be siblings. Since insertions in the three different phage-resistant mutants were observed both in the *trans* gene and in the intergenic region between *trans* and *orf1*, the phage resistance phenotype could not be caused by a knocked-out *trans* gene. Furthermore, the *lin* gene downstream of *orf1* is transcribed in the opposite direction. Taken altogether, these results indicated that *orf1* is a key player in the phage resistance phenotype.

At least two hypotheses could explain the involvement of *orf1* in the phage resistance phenotype. *orf1* could be part of an operon transcribed from a promoter upstream of *trans* and thus terminated by the pGhost9::ISS1 insertions, thereby inactivating transcription of the gene. A second possibility is that the pGhost9::ISS1 insertion provided a promoter upstream of *orf1*. In the latter scenario, transcription of *orf1* would be initiated from a promoter in the ISS1 sequence or from the *Em^r* gene in the pGhost9::ISS1 construct, leading to activation of a phage resistance phenotype. When the insertion mutants were cured of the vector pGhost9::ISS1, leaving a single copy of ISS1 at the integration site, cured *L. lactis* strains JH-49 to JH-52 (Table 1) lost the phage resistance phenotype, suggesting that a promoter activity originated from the vector, possibly from the *Em^r* gene (Fig. 1A).

To test the hypothesis that *orf1* was transcribed from a promoter in the inserted plasmid pGhost9::ISS1, an RT-PCR assay was performed with *L. lactis* strains with and without the insertion. RNA from exponentially growing cells was used as the template for random RT of total RNA, and primers annealing to an internal region of *orf1* were used to amplify the cDNA by PCR. A PCR product of the expected size was observed in the mutant with the insertion (Fig. 2, lane 3) but not in the wild-type strain (Fig. 2, lane 2), indicating that the transcription of *orf1* was turned on in the mutant. A negative control experiment was conducted without reverse transcriptase, and it confirmed that there was no contaminating DNA in the samples (Fig. 2B). A positive control experiment using primers for the glycolytic genes *gapB* and *pfk* was also performed (data not shown). The positive results of this experiment eliminated the possibility of artifacts due to loss of RNA in the samples.

FIG. 2. RT-PCR with RNA isolated from exponentially growing *L. lactis* cultures. Lane 1, JH-20(pJH2); lane 2, JH-54(pLC5); lane 3, JH-32 (insertion mutant); lane C, control PCR with *L. lactis* MB112 chromosomal DNA; lane L, ladder (GeneRuler; 100 to 10,000 bp; Fermentas). (A) Experiment performed with reverse transcriptase. (B) Experiment performed without reverse transcriptase.

A silent phage resistance mechanism is encoded by *orf1* on the chromosome of *L. lactis* subsp. *cremoris* MG1363. To test the hypothesis that *orf1* encodes a phage resistance mechanism, a DNA fragment (positions 1021 to 2320 in the GenBank accession number AF324839 sequence) comprising only one open reading frame (*orf1*) and the upstream region including the most distant insertional mutation site at position 2296 was cloned in the promoterless shuttle vector pCI372 (pJH6) and in the expression vector pLC5 (pJH2) (Fig. 1B). The resulting constructs were transformed into *L. lactis* MB112, and the resulting strains (JH-53 and JH-20, respectively) were tested to determine their phage resistance. *L. lactis* JH-53 (containing pJH6, with no promoter upstream of *orf1*) was sensitive to phage sk1, whereas *L. lactis* JH-20 (containing pJH2, with a strong promoter upstream of *orf1*) was resistant to phage sk1 (Fig. 1B). In fact, the highest level of expression was observed when *orf1* was expressed from the pLC5 vector, which was probably due to the strong P₅₉ promoter (Fig. 2, lanes 1). Furthermore, expression of *orf1* (pJH2) in another host (*L. lactis* subsp. *lactis* IL1403) also conferred a phage resistance phenotype (data not shown). The results described above indicated that expression of *orf1* was responsible for the phage resistance phenotype.

To verify that a protein encoded by *orf1* was responsible for the resistance to phage sk1, a frameshift mutation was introduced into *orf1* by digestion at a unique *Cla*I site (codon 36 in *orf1*) and filling of the ends with the Klenow fragment, followed by ligation, confirmation by sequencing, and transformation of the resulting plasmid, pJH3, into *L. lactis* MB112. The frameshift mutation eliminated the phage resistance phenotype in the resulting strain, JH-24 (Fig. 1B). We concluded that the protein encoded by *orf1* was responsible for the phage resistance phenotype.

Sequence and analysis of *orf1*. The 1,300-nucleotide DNA fragment cloned into pJH2 was sequenced and found to be 100% identical to the region including nucleotides 1021 to 2320 in the GenBank accession number AF324839 sequence, as well as positions 697547 to 698846 in the complete genome sequence of *L. lactis* MG1363 (accession number AM406671). The G+C content of *orf1* was 31.7%. No suitable promoter was found upstream of *orf1* (bp 1 to 430) using Winseq soft-

TABLE 3. EOP and phages tested

Species	Phage	<i>L. lactis</i> strain	EOP ^c
936 ^a	sk1	JH-20(pJH2)	$2.7 \times 10^{-4} \pm 1.4 \times 10^{-4}$
	p2	JH-20(pJH2)	$4.8 \times 10^{-4} \pm 1.8 \times 10^{-4}$
	jj50	JH-20(pJH2)	$8.3 \times 10^{-5} \pm 0.5 \times 10^{-5}$
	712	JH-20(pJH2)	1.1 ± 0.2
	P008	JH-22(pJH2)	$3.8 \times 10^{-4} \pm 1.5 \times 10^{-4}$
	bIL170	JH-22(pJH2)	$3.1 \times 10^{-4} \pm 1.2 \times 10^{-4}$
c2 ^a	c2	JH-20(pJH2)	$5.2 \times 10^{-5} \pm 0.4 \times 10^{-5}$
	bIL67	JH-20(pJH2)	$2.0 \times 10^{-4} \pm 1.2 \times 10^{-4}$
	ml3	JH-20(pJH2)	$3.4 \times 10^{-4} \pm 0.3 \times 10^{-4}$
	eb1	JH-20(pJH2)	$2.2 \times 10^{-4} \pm 0.7 \times 10^{-4}$
P335 ^b	ul36	JH-23(pJH2)	1.0
	ul36.k1t1	JH-23(pJH2)	1.6
	φ31	JH-23(pJH2)	1.0
	φ50	JH-23(pJH2)	1.0
	Q33	JH-23(pJH2)	0.7
	Q30	JH-23(pJH2)	0.8
	P335	JH-23(pJH2)	0.4

^a The EOP of 936- and c2-like phages is 1.0 on *L. lactis* MG1363 (MB112) and MB112(pLCS) (JH-54). The EOP of phages P008 and bIL170 is 1.0 on *L. lactis* IL1403.

^b The EOP of P335 species is 1.0 on *L. lactis* SMQ-86.

^c The EOP was calculated by determining the average for three independent assays.

ware (F. G. Hansen, unpublished data). This is in agreement with the experimental results showing that *orf1* is silent in wild-type *L. lactis* strain MG1363. The translation start codon of *orf1* was preceded by a suitable RBS (5'-TGAACGGAGA G-3'; the underlined nucleotides match the consensus sequence). DNA sequence analysis did not result in identification of any transcription terminator structures between *orf1* and the upstream *trans* gene, leaving the possibility that *orf1* could be part of an operon initiated upstream of *trans*. However, the negative RT-PCR results for *orf1* transcription in the wild-type strain (Fig. 2, lane 2) suggest that *orf1*, and perhaps *trans* as well, is not expressed under the conditions tested. This was confirmed by a Northern analysis of the insertion mutant *L. lactis* JH-32 and *L. lactis* wild-type strain MG1363, in which transcription of *orf1* was observed only in JH-32 (data not shown).

The phage resistance mechanism encoded by *orf1* is effective against virulent lactococcal phages belonging to the 936 and c2 species. Representatives of the three main lactococcal phage species, 936, c2, and P335, were tested to determine their sensitivities to *orf1* (Table 3). EOPs of approximately 10^{-4}

were obtained for the four c2-like phages tested and for five of the six members of the 936 species tested (Table 3). Phage 712 (936 species) was not sensitive to *orf1* (EOP, 1) (Table 3). The seven phages belonging to the P335 species were also not affected by the presence of *orf1* (Table 3). Because the efficacy of *orf1* with virulent P335 phages was tested using another *L. lactis* host (SMQ-86), we needed to rule out the possibility that the insensitivity of P335 phages was due to a mutation in pJH2 present in *L. lactis* SMQ-86 (*L. lactis* JH-23). Thus, plasmid pJH2 was isolated from *L. lactis* JH-23 and reintroduced into *L. lactis* MB-112. Phage p2 was unable to effectively replicate on this recombinant host (EOP, 10^{-4}), indicating that pJH2 was indeed functional and confirming that the P335 phages were not sensitive to *orf1*.

The EOPs of 936- and c2-like phages were also determined at 30 and 37°C and were found to be similar, indicating that the system is stable in this temperature range. EOPs of $1.6 \times 10^{-5} \pm 0.2 \times 10^{-5}$ and $2.7 \times 10^{-4} \pm 0.3 \times 10^{-4}$ were obtained for phages sk1 and p2, respectively, when *orf1* was expressed from the chromosome in the insertion mutants. These values are in the range obtained when *orf1* was expressed from a strong promoter in the low-copy-number expression vector pLCS5. However, when the insertion mutants were tested with phage c2, EOPs of $4.6 \times 10^{-1} \pm 0.9 \times 10^{-1}$ were obtained, indicating that a higher level of expression of *orf1* is required with c2. Finally, pJH2 was transformed into *L. lactis* MG1363 (JH-79), and the EOPs with phages p2, 712, and c2 were determined. EOPs similar to those obtained with MB112 eliminated the possibility that the *upp* deletion in MB112 influenced the phage resistance phenotype.

The efficiency of *orf1* as a phage resistance mechanism was further characterized using phage p2 and *L. lactis* strains JH-20 (pJH2) and MB112 (Table 4). The efficiency of formation of centers of infection of phage p2 with *L. lactis* JH-20 was $0.5\% \pm 0.2\%$, indicating that only 5 of 1,000 infected cells released at least one virulent phage. One-step growth curve experiments were then performed in the presence or absence of pJH2 (*orf1*), and the burst size was reduced by 72% (from 38.8 ± 5.7 in MB112 to 11.1 ± 5.2 in JH-20) (Table 4).

The phage resistance system encoded by *orf1* is an *Abi* mechanism. A series of experiments were conducted to determine the type of phage resistance mechanism encoded by *orf1*. Adsorption assays showed that the level of adsorption of phage sk1 to phage-resistant cells was $95.9\% \pm 10.6\%$, which is similar to the adsorption level for the wild-type host. Furthermore, fluorescently labeled (SYBR gold-labeled) phage DNA was

TABLE 4. Effect of *AbiV* on phage p2^a

Strain	EOP ^b	Efficiency of formation of centers of infection (%) ^c	Burst size (PFU/cell) ^d	Latency time (min) ^d	Cell survival ^e (%)	DNA replication ^f	Plaque size (mm)
MB112 (wild type)	1.0	100	38.8 ± 5.7	20–30	$6.1 \times 10^{-5} \pm 1.3 \times 10^{-5}$	+	1.5 to 1.7
JH-20 (<i>AbiV</i>)	$4.8 \times 10^{-4} \pm 1.8 \times 10^{-4}$	0.5 ± 0.2	11.1 ± 5.2	20–30	$3.1 \times 10^{-6} \pm 0.3 \times 10^{-6}$	+ (concatemeric)	Pinpoint to 0.7

^a All assays were performed at 30°C.

^b The EOP of phage p2 on *L. lactis* MG1363 (MB112) is 1.0 ($n = 3$).

^c The MOI was 0.5 ($n = 3$). The efficiency of formation of centers of infection of phage p2 on *L. lactis* MB112 is 1.0.

^d The MOI was 0.2 ($n = 3$).

^e The MOI was 5 ($n = 3$).

^f The MOI was 2 ($n = 2$).

FIG. 3. Replication of phage p2 DNA during infection of phage-sensitive strain *L. lactis* JH-54 (A) and phage-resistant strain *L. lactis* JH-20 (B). Samples were taken at -10, 0, 10, 20, 30, 40, 50, 60, 90, and 120 min, and total DNA was restricted with EcoRV. The band at 5.3 kb (consisting of the bands at 1.3 and 4 kb for mature resolved DNA) spans the *cos* sites which mark the extremities of the phage genome. The numbers in panel C indicate the sizes of phage p2 DNA fragments after EcoRV digestion and heat treatment. The ladder used was GeneRuler (100 to 10,000 bp; Fermentas).

visualized after sk1 infection of the phage-resistant strain *L. lactis* JH-20 and the phage-sensitive strain MB112. Immediately following phage infection, a fluorescent halo of adsorbed phages was seen surrounding the host cells (data not shown). Less than 15 min after the beginning of phage infection, the fluorescent signal on the cell surface was reduced, while a bright fluorescent signal was observed in the center of the cell, indicating that the phage DNA had been ejected into the host cell (data not shown). Identical results were obtained for phage-resistant and phage-sensitive strains. The results described above indicated that the phage resistance mechanism was not an adsorption- or ejection-blocking mechanism.

Smaller phage plaques were observed at a frequency of 10^{-4} with *L. lactis* JH-20, which contained *orf1*. These plaques were propagated on *L. lactis* JH-20 cells, as well as on the phage-sensitive host *L. lactis* MG1363, and were found to be insensitive to the antiphage mechanism, which demonstrated that this mechanism is not a restriction/modification system. A cell survival assay showed that there was virtually no survival of cells of the sensitive strain or the phage-resistant strain upon phage infection (Table 4). Moreover, the few plaques of phage p2 arising at a frequency of 10^{-4} were smaller on the phage-resistant strain than on the wild-type sensitive *L. lactis* strain (Table 4). The characteristics described above are well-documented characteristics of abortive phage infection mechanisms. The low G+C content of *orf1* (31.7%) is also typical of Abi mechanisms. Taken altogether, the data show that the phage resistance mechanism encoded by *orf1* is an abortive infection mechanism. Accordingly, the gene was designated *abiV* and the Abi mechanism was designated AbiV.

Analysis of the AbiV protein. AbiV consists of 201 amino acids and has a calculated molecular mass of 22,692 Da. A His-tagged AbiV protein was overexpressed in *E. coli* and purified. The native molecular mass of the purified AbiV protein was estimated to be 49 ± 0.3 kDa by gel filtration (data not shown), suggesting that AbiV forms a dimer in its native form. The pI was calculated to be 5.37. The protein does not contain any putative transmembrane or signal peptide motifs, and it is

therefore likely to be cytosolic. Although 69% of AbiV consists of α -helices, no DNA-binding helix-turn-helix motif was found in AbiV. Similarity searches using several bioinformatics tools did not reveal any similarity to other lactococcal proteins or any other phage resistance mechanism, nor was AbiV found in other sequenced genomes of lactococcal strains. Likewise, no conserved domains were found in the protein.

Expression of AbiV does not affect the cellular growth rate or final biomass. A cell growth experiment was conducted to test whether the expression of AbiV from a strong plasmid-encoded promoter could influence the cell growth rate or the final biomass. *L. lactis* JH-20 (Abi⁺) and MB112 (Abi⁻) grew exponentially in GM17 at 30°C with growth rates of 1.04 ± 0.08 and 1.00 ± 0.03 h⁻¹, respectively. The final concentrations were $2.49 \times 10^9 \pm 0.10 \times 10^9$ and $2.45 \times 10^9 \pm 0.02 \times 10^9$ cells ml⁻¹, respectively. Based on these experiments, it was concluded that the expression of AbiV did not affect cellular growth. Moreover, the results showed that the newly constructed expression vector pLC5 did not interfere with cell growth.

AbiV affects phage DNA maturation. The DNA replication of the *cos*-type virulent lactococcal phage p2 was determined at time intervals during infection of a resistant *L. lactis* strain (JH-20) and a sensitive *L. lactis* strain (JH-54). Phage DNA was analyzed by digesting the total DNA isolated from infected cells with EcoRV and then comparing the resulting fragments with the EcoRV restriction map of phage p2 (Fig. 3). Ten minutes after infection, replication of phage DNA was observed in both strains. In sensitive cells, the concentration of phage DNA decreased around 40 min after infection, coinciding with lysis of the host culture. In comparison, phage DNA persisted in the resistant cells throughout the experiment, which was terminated after 2 h.

Analysis of the EcoRV-digested phage DNA pattern revealed (among other bands) two bands at 1.3 and 4 kb, as well as a 5.3-kb fragment in the phage-sensitive culture. The 5.3-kb DNA fragment spanned the *cos* site on the phage p2 genome, and the 1.3- and 4-kb fragments represented the mature en-

capsidated phage DNA. Therefore, both replicative DNA and encapsidated DNA were observed in the phage-sensitive strain due to continuous DNA replication throughout the phage lytic cycle and simultaneous encapsidation of mature DNA. With the resistant cells, only the 5.3-kb fragment was obtained. The absence of the 1.3- and 4-kb bands, as well as the presence of phage DNA throughout the experiment, indicated that phage DNA accumulated in its concatemeric (nonmature) form in the resistant *L. lactis* cells. Similar results were obtained with the closely related phage sk1 (data not shown). The results described above showed that *AbiV* prevented cleavage of the replicated phage DNA and thus that it acts at a later stage of the phage infection process.

DISCUSSION

Insertional mutagenesis was used to confer phage resistance to *L. lactis* subsp. *cremoris* MG1363. Four phage-resistant mutants obtained in three independent insertional mutagenesis experiments were selected for further analysis, and in all cases the insertion was located in the same 350-bp region of the bacterial chromosome. Insertional mutagenesis with the pGhost9::ISS1 system (48, 49) was previously used to knock out factors involved in phage adsorption (24) and in DNA ejection (47). Such loss-of-function effects of insertional mutagenesis are the usual outcome of experiments like these (24, 67, 75). However, using a similar strategy, Luccini et al. also isolated a phage-resistant mutant of *Streptococcus thermophilus* in which promoter activity from the insertion turned on a downstream restriction/modification mechanism (47). A similar finding was obtained in this study as integration of pGhost9::ISS1 conferred phage resistance to the cells by activating an abortive infection mechanism.

All the ISS1 inserts were oriented in the same direction as *orf1*, which made us speculate that *orf1* could be transcribed from a promoter within ISS1 or from the *Em^r* gene in pGhost9::ISS1 (49). Promoter activity has previously been reported outward of insertion sequences (29) in both the forward (24) and backward (15) directions, and insertion sequences with a high degree of similarity to ISS1 have also been shown to be in the vicinity of abortive infection mechanisms (4, 15). Searches for promoter sequences in the forward direction of ISS1 were unsuccessful. This observation was supported by the reversion to a phage-sensitive phenotype when mutants were cured of the plasmid while they kept a single copy of ISS1 at the integration site (Fig. 1A). However, the activation hypothesis was supported by the results of subsequent transcription analyses using RT-PCR with mRNA from *L. lactis* strains with and without the insertion. Transcription of *orf1* was observed in the presence of an upstream pGhost9::ISS1 insertion. These results indicated that *orf1* was indeed turned on by a promoter in pGhost9::ISS1, which was most likely the promoter of the *Erm* gene. Cloning of *orf1* and its upstream region into the promoterless vector pCI372 failed to confer resistance, while similar cloning into the expression vector pLC5 resulted in a phage resistance phenotype. An active *orf1* was later shown to encode the abortive infection protein *AbiV*, confirming that integration by pGhost9::ISS1 can be used to transcribe silent genes on bacterial chromosomes.

The ISS1 insertion sequence has been reported to integrate

randomly in the chromosome of *L. lactis* (49). In the present study, ISS1 integrated in a 350-bp region in three independently obtained mutants, conferring phage resistance. In a similar study with *L. lactis* subsp. *cremoris* Wg2 and *L. lactis* subsp. *lactis* IL1403, Dupont et al. (24) obtained phage-resistant mutants that were due to a defect in phage adsorption with frequencies around 5×10^{-6} , the same frequency obtained in the present study. We did not obtain any mutants with reduced phage adsorption, which indicates either that the phage receptors on *L. lactis* MG1363 are more difficult to mutate and perhaps are essential for cell growth or that ISS1 integrates in a nonrandom manner in this strain.

abiV is located in a 59-kb DNA region of the *L. lactis* MG1363 chromosome (nucleotides 657,000 to 706,000), which was previously referred to as an "integration hot spot" (77) because it contains DNA sequences involved in DNA mobility. For example, this region contains genes and sequences usually found on plasmids, as well as almost 20% of the 71 insertion sequences found in the 2,529,478-bp genome of MG1363. Interestingly, two of these insertion sequences, namely IS946 and IS1297, share 98 and 85% nucleotide similarity with ISS1, respectively. There is only one copy of each of these insertion elements in the genome of *L. lactis* MG1363. The high frequency of insertion elements in this region suggests that it has features favoring the integration of insertion sequences in general and of ISS1-type sequences in particular. Thus, ISS1 integration may not be completely random in *L. lactis* MG1363. The location of *abiV* in the integration hot spot region among remnants of plasmid DNA and insertion sequences also makes it tempting to speculate that *abiV* originated from a plasmid. This would correspond to the observation that most of the previously described lactococcal *Abi* systems are encoded on plasmids (2, 14, 28, 73).

abiV conferred phage resistance when it was cloned in an expression vector, but it lost this function upon introduction of a frameshift mutation into the gene. Cells expressing *AbiV* exhibited typical abortive infection characteristics upon phage infection, such as normal phage adsorption and DNA ejection, no restriction/modification activity, and cell death. Very few infected cells released progeny phages, and the burst size was also reduced. Since database searches revealed no similarity to any other phage resistance mechanism, it was concluded that *AbiV* is a novel *Abi* mechanism.

AbiV inhibits proliferation of small isometric phages belonging to the 936 species and of prolate phages belonging to the c2 species, but it has no effect on small isometric phages belonging to the P335 species. This range of efficacy against the three main phage groups has been observed with other lactococcal *Abi* mechanisms (4, 14, 26, 53, 65, 66), but the lack of similarity with other *Abi* proteins suggests that there is a different mode of action. Combinations of diverse phage resistance mechanisms are often observed for plasmids isolated from wild-type strains of *L. lactis* that are highly resistant to phages (2, 21, 25, 32). Since *AbiV* is a novel *Abi* mechanism, it may be suitable to use it in combination with other phage resistance mechanisms to confer efficient phage resistance to industrial strains of *L. lactis*.

Lactococcal *Abi* mechanisms are often characterized further by identifying the general step in the phage lytic cycle that is inhibited (14). In infected *AbiV*-containing cells, we observed

that phage DNA is replicated but maturation is halted, resulting in accumulation of concatemeric phage DNA. Replication of phage DNA indicated that transcription and translation of early phage genes took place in the presence of AbiV.

It was also observed that the efficiency of AbiV activity (expressed as EOP) against 936 phages was not improved by expressing *abiV* from the strong lactococcal promoter P₅₉ (76) in a low-copy-number plasmid (68) compared to expression from an internal promoter in pGhost9::ISS1 and expression of a single copy from the chromosome. This suggests that only small amounts of AbiV are needed or that competitive inhibition of a substrate is not part of the mode of action of AbiV. However, the expression level was important for the efficiency of activity against c2 phages. Toxicity of Abi proteins has been demonstrated for AbiD1, AbiK, AbiN, and AbiO (14), and in at least two cases, it was associated with regulation of Abi gene expression (4, 27). The absence of effects on the cellular growth rate during high levels of expression of *abiV* demonstrates that AbiV is not toxic to the cell. The absence of sequence similarity between Abi proteins (10, 14) makes it desirable to obtain structural data for Abi proteins in order to investigate possible correlations between structure and function. The native multimer state of a protein might be a basic parameter to compare Abi proteins. However, except for motifs in AbiA which are putative multimerization sites (22), AbiV is the only Abi mechanism for which the native state (dimer) has been determined.

In conclusion, AbiV is a novel abortive infection mechanism that was discovered on the chromosome of *L. lactis* subsp. *cremoris* MG1363. This discovery was possible due to transcription from a promoter within pGhost9::ISS1, which was integrated in the upstream region. AbiV is effective against 936 and c2 phages, but the mode of action of this 23rd lactococcal Abi system needs to be investigated further as it may involve an early transcribed phage gene or gene product.

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